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Endoplasmic reticulum bodies: solving the insoluble

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Plant cells produce and accumulate insoluble triglycerides, proteins, and rubber that are assembled into inert, ER-derived organelles broadly termed as ER bodies. ER bodies appear to originate from tubular ER domains that are maintained by cytoskeletal interactions and integral ER proteins. ER bodies sequestering insoluble substances usually are transferred to the vacuole but sometimes remain as cytoplasmic organelles. Some otherwise soluble ER-synthesized proteins are converted to insoluble aggregates to produce ER bodies for transfer to the vacuole. This process constitutes an alternate secretory system to assemble and traffic transport-incompetent insoluble materials.

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Insoluble problems, an introduction

Many substances accumulated by plants, particularly in seeds, are insoluble and present a range of problems for their assembly within an aqueous environment and their transfer to locations distal from their site of synthesis. These insoluble materials are often reserve metabolites such as oil, protein, and rubber. They are produced by the endoplasmic reticulum and marshaled into ER-derived organelles, termed ER bodies. ER bodies may exist as cytosolic organelles but more often function to transport insoluble substances to vacuoles. Insoluble protein assemblies are a means to concentrate large masses of specialized proteins delivered to the vacuole as enzyme precursor or storage proteins. Recent data have given a new perspective on the ER and ER bodies as a plant specific alternate secretory pathway that permits plant cells to produce and use insoluble substances.

Domains of the soluble and the insoluble

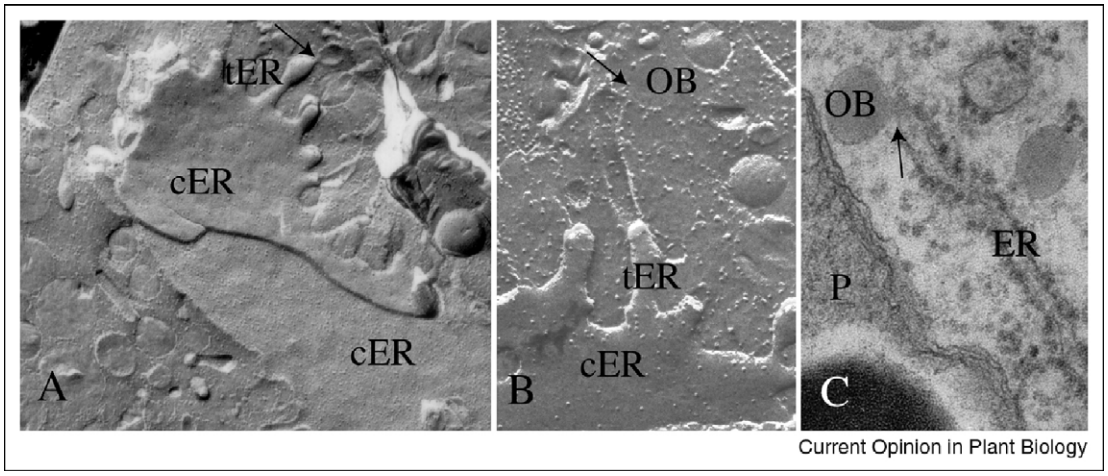
The ER is a dynamic pleomorphic organelle that is the site of synthesis of soluble secretory proteins, the endomembrane's own proteins, and lipids, as well as insoluble triglyceride (TAG), protein, and rubber. Electron microscopy shows that the plant ER is a patchwork of structurally diverse and functionally distinct domains interconnected by a contiguous lumen [1]. The ER comprises cisternal (cER) and tubular ER (tER), the nuclear envelope, plasmadesmata, and a diverse group of ER-derived organelles containing accreted substances, such as oil bodies (OB), protein bodies (PB), protein precursor vesicles/precursor accumulation vesicles (PPV, or PAC), and rubber bodies (RB) [2]. Electron microscopic images of seed ER using thick section heavy metal impregnation [3] and freeze fracture [4] (Figure 1A and B) show interconnected cER sheets and tER projections. The seed tER forms both the OBs (Figure 1A–C) and PBs (Figure 2) and mediates the production of cargo vesicles carrying soluble secretory proteins targeted to the Golgi and on to the storage vacuole or cell surface.

Cytoskeleton frames ER body production

Animal cell tER aligns with the microtubule cytoskeleton, which specifies ER morphology [5]. Disruption of microtubules produces large-scale morphological changes in tER. Components that specify tER interaction with microtubules include the microtubule motor kinesin-1 that drives changes in tER morphology. The tER is connected to the microtubule network by CLIMP-63, a cytoskeleton-linking ER membrane protein that is excluded from the contiguous nuclear envelope [6]. Other proteins that have cytoskeleton interactions with the tER network include an EF hand Ca^{2+} binding protein p22 [7], huntingtin, and kinectin [5].

Plant cell tER aligns with the actin cytoskeleton [8], which suggests its role in establishing of the tER morphology. Actin depolymerization does not disrupt the pre-existing ER structure, only its further modification [9••]. The actin cytoskeleton mediates the localization and transport of RNA transcripts targeted to the tER. For example rice storage protein transcripts exhibit differential distribution. Transcripts encoding glutelin, a soluble vacuolar storage protein, are present on the cER, while transcripts encoding the insoluble prolamin storage protein are associated with tER [10]. Transcripts encoding prolamin move unidirectionally on the cytoskelton to the tER responsible for forming PBs; trafficking is disrupted by cytochalasin D and latrunculin B [11]. The prolamin transcripts bind to a cytoskeletal-associated protein, OsTudor-SN. Silencing the OsTudor-SN repressed both prolamin protein synthesis and transcript abundance, decreasing the number of PBs assembled [12••]. These data indicate that translation of prolamin

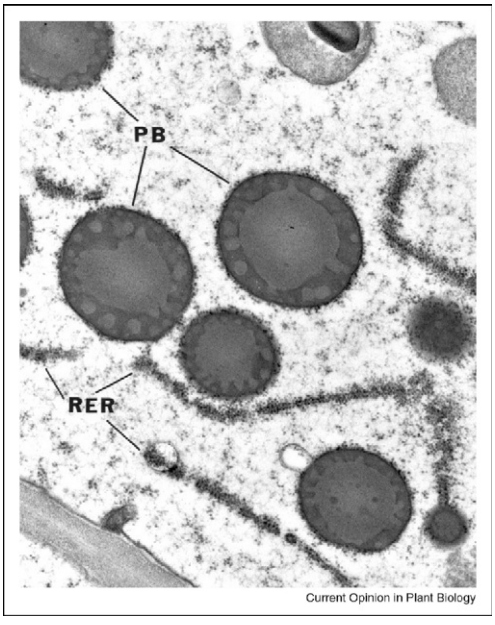
Figure 1



The formation of soybean oil bodies (OB) by the tubular ER (tER) as observed by freeze fracture replica **(A and B)** [3] and conventional thin-section electron microscopy **(C)** is shown. Numerous tER extensions radiate from the cisternal ER (cER) with the OBs assembled and released from the tip of tER (arrows).

transcripts occurs at site of protein accumulation so that the specific receptors that recognize tER domain specific transcripts and direct the synthesis and assembly of these hydrophobic proteins at the site of PB ontogeny may be discovered. Further experiments are needed to establish whether this path for PB ontogeny extends to other ER bodies. Figure 3 illustrates the interpreted relationship between the cytoskeleton, transcripts, tER, and ER bodies.

Figure 2



Maize zeins and PBs possess a complex structure shown in this electron micrograph with the more hydrophobic zeins in the PB matrix interior and the more hydrophilic zeins in the peripheral matrix. While most if not all other PBs are assembled coordinately with the matrix protein synthesis, maize PBs are assembled by first synthesizing the hydrophilic gamma zein and then later synthesizing the hydrophobic alpha zeins. The co-assembly of the proteins results in PB formation with subdomains containing different zeins. The maize zein PB remains attached to the ER as a subdomain. Micrograph provided by Dr Brian Larkins, Univ of Arizonia.

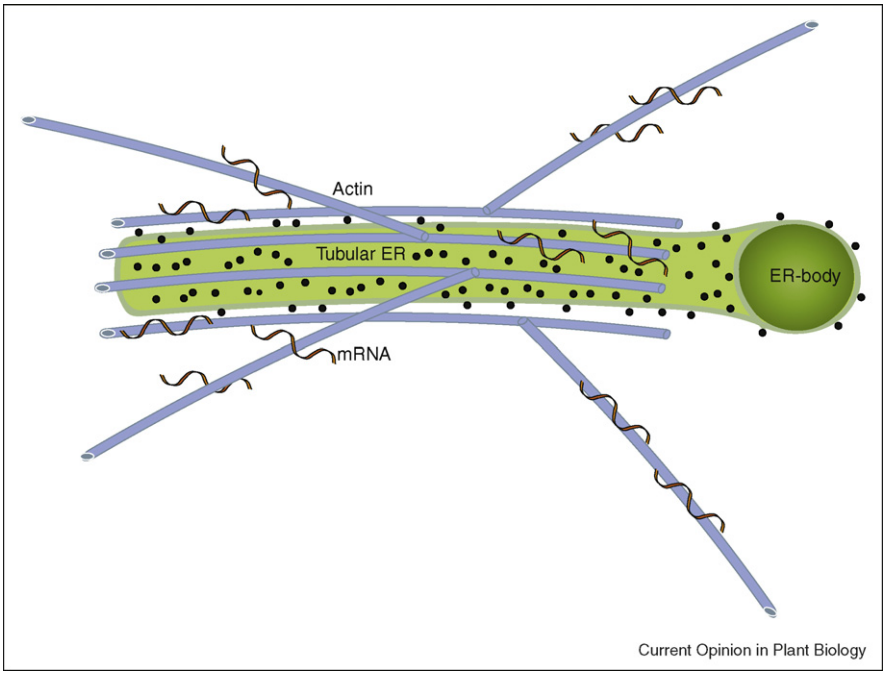
Tube bending specifies tER morphology

A family of proteins, the reticulons, has been identified to provide the force to bend the ER into tubes [13]. Reticulon–reporter fusions expressed in plant cells localize in the tER. Reticulons contain two *trans*-membrane domains separated by a cytoplasmic facing loop. The *trans*-membrane domains form a loop asymmetrically embedded in the ER membrane that imposes a curvature so that the inner half of the ER bilayer bends more than the outer half creating the tER conformation. The *Arabidopsis* genome contains 21 members of the reticulon family [14••]. Sixteen of the *Arabidopsis* reticulons possess a consensus ER di-lysine retention/retrival sequence [15]. There are three distinct types of reticulon proteins [14••], which suggests that they serve specialized functions during tER and ER body formation. Consistent with this suggestion, overexpression of one *Arabidopsis* reticulon massively remodels the ER by increasing the distribution and constrictions of the tER [16••].

Separating oil from ER

The storage of TAG oil reserves has antecedents in unicellular prokaryotic and eukaryotic organisms [17]. The template for OB ontogeny has persisted throughout evolution, with the plant, animal, and fungal kingdoms each producing ER-derived lipid bodies or OBs consisting of a TAG core encased in a phospholipid monolayer that often is embedded with one or more unique surface proteins. Triglycerides are synthesized by ER-localized diacylglyceride transferase (DGAT), which in plants

Figure 3



An interpretation of the morphology of the key features of a tER segment assembling an ER body is shown. The tER aligns with an actin cytoskeleton framework that defines the morphology of the tER segment as well as functioning as a transport track to move specific transcripts to tER. Not shown in the diagram are reticulons, the tER integral membrane proteins that define the structure of the tER. Coupled translation of the ER body specific transcripts and accretion of triglyceride, protein, or rubber cargo forms the ER body. The ER body accumulates the accreted insoluble until a critical size is attained after which it detaches from the ER releasing a mature ER body that comprises a cargo core encased in an ER-derived membrane. The ER body membrane is either an ER bilayer with attached ribosomes when the organelle has a protein cargo, while the ER body membrane is a phospholipid monolayer that may include ER body specific membrane proteins when the cargo is triglyceride or rubber. The absence of ribosomes on the OB or rubber body membrane is the consequence of the monolayer membrane that will exclude ribosome-binding proteins that require a bilayer structure.

exists in two distinct forms, DGAT1 and DGAT2 [18]. The seed TAG core co-assembles with surface proteins termed oleosins, which have a three-domain structure and a conserved 70+ amino acid hydrophobic central domain [19]. Other minor oil body constituents include caleosin [20] and steroleosin [21], whose function and ubiquity remain to be established. ER-synthesized TAG accretes to form membrane surface patches [22] that have been visualized during lipid body ontogeny in animal cells [23•]. Electron microscopy revealed that OBs originate from the tER's distal tip by splitting the bilayer, with the OB forming from the cytoplasmic half of the ER bilayer.

Recent studies have begun to define the molecular processes that underlie the microscopic observations of OB ontogeny. Tung seed DGAT 1 and 2 have been localized to different ER domains using epitope-tagged proteins expressed in BY2 cells [24••]. DGAT1 possesses seven *trans*-membrane domains, while DGAT2 mimics oleosin structure by having a single *trans*-membrane loop consisting of two *trans*-membrane domains, with the carboxy-terminal domain facing the cytoplasm. The single loop domain of DGAT2 could orient in the tER, consistent with its discrete localization in BY2 cells. Oleosin is cotranslationally inserted into ER, although the oleosin central hydrophobic domain is physically too large to stably insert in an ER bilayer. Modification/expression experiments have indicated that oleosin must have a 'relaxed' cotranslational insertion conformation [25], where the hydrophobic domain lies within the core of the tER bilayer. If the DGAT2 and oleosin transcripts were directed at the same tER domain, this would result in co-synthesis of TAG and oleosin. This could result in a phase separation of the TAG from the ER and partition of oleosin into the incipient OB by hydrophobic interactions. Caleosin has been speculated as having a role in the formation and mobilization of OBs. In maturing barley seeds, caleosin is accumulated coordinately with TAG, but in a microscopic assay oleosin and caleosin have a different, somewhat overlapping distribution [26]. These results suggest that caleosin may play a transient role in the formation of OBs, but not necessarily become incorporated into the mature OB membrane. One of the curious features of DGAT2, caleosin, and oleosin is that each has a single membrane insertion loop, but of varying length (oleosin > caleosin > DGAT2). This suggests

that one protein could be displaced by another via protein–TAG hydrophobic interactions, with caleosin playing a role to facilitate assembly. Interestingly, Tung seed DGAT2 penetrates the ER membrane with a small loop in the lumen that connects the two *trans*-membrane domains, whereas caleosin and oleosin apparently do not penetrate the membrane. This structural feature would facilitate DGAT2 displacement from the incipient oil body by TAG/protein hydrophobic affinity.

One test of this concept is to suppress oleosin and examine the effect on OB ontogeny. One can predict that without oleosin to displace other proteins, TAG will remain bound to tER proteins and aberrant OBs could result. Oleosin RNAi and insertion mutants have been studied in *Arabidopsis* [27••] and soybean [28••] and result in the accumulation of giant oil bodies in mature seeds. This result supports the proposal that oleosins impede coalescence during desiccation. In immature seeds, ends of the tER in a soybean oleosin RNAi plant produce 50 nm micro OBs that form increasingly larger and then giant OBs, demonstrating that oleosins are important in OB ontogeny [28••]. Soybean oleosin RNAi also results in the formation an ER/OB complex enriched in caleosin, a result that indicates this protein is trapped by production of aberrant OBs. Caleosin has a cytoplasmic EF hand Ca^{2+} binding domain that may be the functional equivalent of the EF hand Ca^{2+} binding protein, p22, involved in animal tER–cytoskeleton interactions. Oleosin silencing, with the resulting increase in caleosin, suggests that additional protein factors are necessary to produce seed oil bodies.

Insoluble proteins and ER bodies

Plants make broad, although limited use, of protein accretion in the ER to produce PBs that sequester storage proteins precursors [29], cysteine proteases and other enzymes, and cereal prolamins. Once formed most PBs are transferred to the vacuole by a direct ER–vacuole trafficking (ERVT) route that bypasses the Golgi [2]. This pathway gives plants the capacity to traffic insoluble transport-incompetent protein accretions. While triglycerides and rubber polymers are hydrophobic and therefore self-accreting, with the exception of cereal prolamin storage proteins other PB proteins are soluble and should be transport competent. Yet, plants have evolved post-synthetic processes that assure some secretory proteins aggregate to produce PBs that are transferred to the vacuole via ERVT.

The insoluble protein matrix

Although considered typical PBs, the hydrophobic cereal prolamins may actually be more of an exception among the proteins in ER bodies. Prolamins accrete in the ER with the assistance of ER chaperones, including BiP and PDI. Wheat gliadin monomers assemble into large oligomers in the ER by forming disulfide bridges between the monomers before accreting in PBs that are subsequently transferred to the vacuole by autophagy [30]. Maize prolamin PBs have a complex structure with four distinct types of proteins, α-,β-, γ-, δ-zeins, differentially distributed into different domains within the PB. The 22 kDa α-zeins interact with a lumen-facing ER membrane protein identified by the *Floury1* mutation, *Fl*1 [31••], may chaperone these proteins to facilitate their spatial distribution within the PB assuring that 22 kDa α-zeins are localized in the PB interior as a ring around the hydrophobic 19 kDa α-zein core. In the absence of *Fl*1, the 22 kDa zeins are randomly distributed in the PB matrix showing that the *Fl*1, protein influences in the assembly of the PBs. Maize PBs form at the distal end of tER, but unlike other ER bodies the maize PBs remain attached to the ER [32].

Do PBs avoid quality control?

For most eukaryotes the accretion of proteins in the ER, whether from genetic disease inducing the formation of cellular structures such as Russell Bodies [33] or from other stresses, results in cellular responses to mitigate the problems produced by accumulations of accreted proteins. This response, termed as quality control (QC) and unfolded protein response (UPR), leads to a cascade of events that result in degradation of the accreted protein by retrograde transport from the ER [34]. In an alternate disposal mechanism, ER accreted protein is transferred to the lysosome/vacuole by either endomembrane progression through the Golgi, if transport competent, or more often by autophagy by lysosomes/vacuoles of the ER-included protein accretion, where it is degraded. Plant PBs appear to be an exception to QC/UPR, but not necessarily for vacuolar degradation. The accretion of prolamins does not result in protein degradation by QC and UPR, although these mechanisms are present in seeds and function with the induction of ER-stress proteins during the synthesis of zeins and [35] other protein accretions. Two maize starchy endosperm (opaque) mutants that induce UPR have been shown to result from malformed zeins; one has an altered signal peptide sequence [36] and the other a frame shift mutation that caused a nonsense protein [37••]. These mutants show that wild-type zeins receive a pass from QC/UPR while malformed zeins do not. Wheat and rice prolamin PBs are transferred to the vacuole by ERVT where the storage protein accumulates. By contrast, zein PBs are transferred to the vacuole in transgenic seed PSVs and are degraded [38].

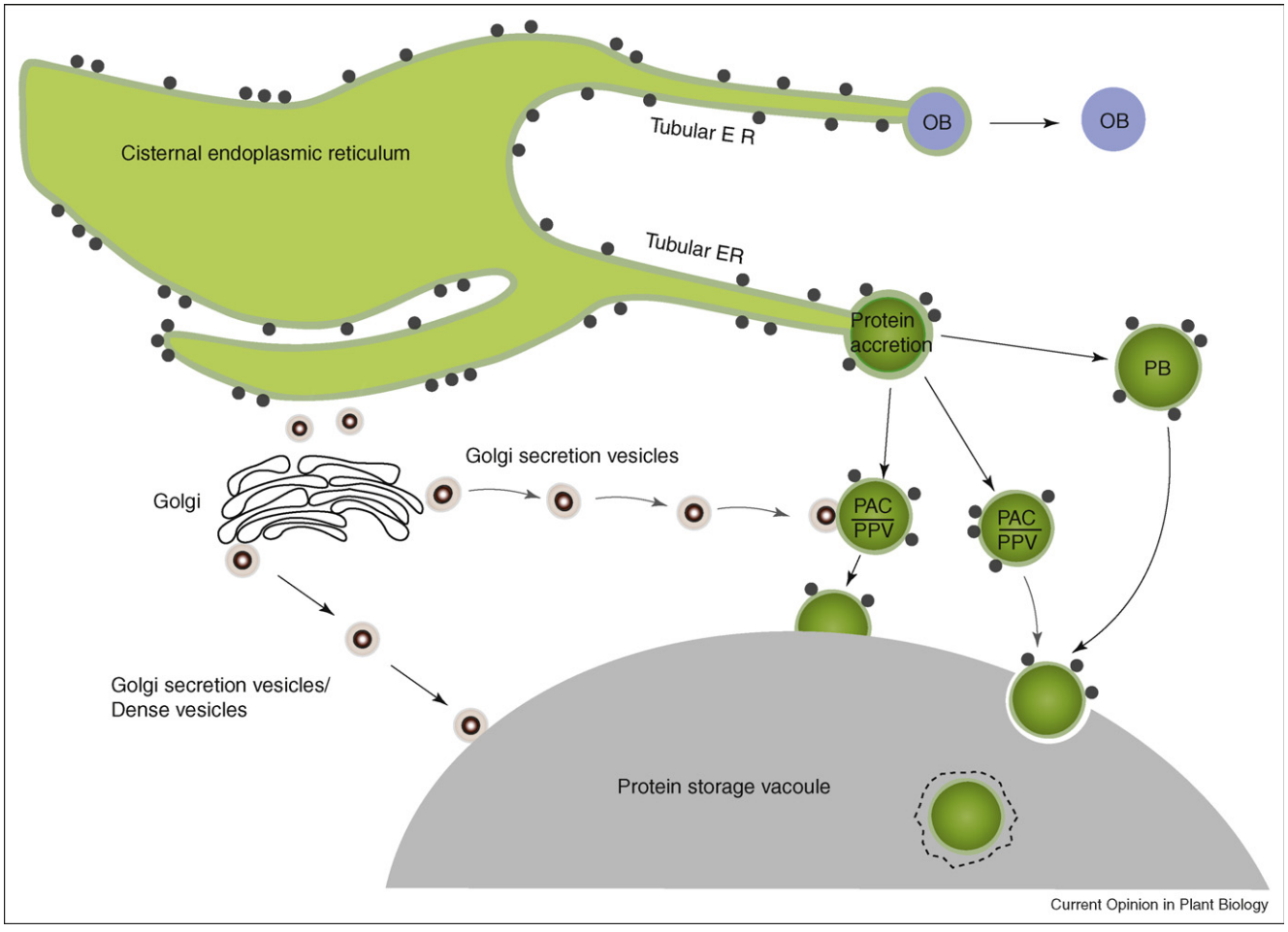
Making the soluble insoluble and then the insoluble soluble

PB bodies are used in plants as a means to deliver proteases and seed storage protein precursors to the vacuole by ERVT. What is remarkable about these classes of protein is that they are soluble, transport-competent proteins that possess vacuolar-targeting

sequences to traffic them, using endomembrane progression by the ER–Golgi–vacuole route. In addition to these proteins, a large number of soluble engineered reporter and transgene proteins can be induced to accrete by adding an ER-retention sequence K/HDEL [39]. The addition of K/HDEL retards protein progression from the ER [40]. In many instances these proteins will be accreted in PBs, where their posttranslational stability is enhanced [41]. This raises an interesting biological question: why are some soluble transport-competent proteins converted to insoluble transport-incompetent proteins that follow a secondary ERVT route to the vacuole. Unfortunately, there are few answers, but existing data provide hints and directions for future inquiry.

Soluble vacuolar protein precursors that are known to be sequestered in PBs include cysteine proteases: a RD21, vacuolar processing enzyme [42], a family of KDEL-tailed proteins [43], and storage protein precursors [44,45] that are components of the protein storage vacuole crystalloid. For example, cysteine protease precursors possess vacuole-targeting sequences within the precursor domain, which indicates they should be recognized by the Golgi-localized vacuolar-targeting receptor if the protein moved to the vacuole instead of being diverted into PBs and ERVT [2]. That these proteins form PBs for ERVT is one possible hint of function. Both cysteine proteases and crystalloid proteins need to be delivered at high concentrations, the proteases to mobilize reserve proteins or to turn over cellular constituents after stress and for the crystalloid proteins to form a high concentration to enhance the formation of a storage vacuole matrix subdomain. By accreting a single type of protein into PPV/PAC PBs and delivering the protein as large

Figure 4



A diagrammatic representation of the ontogeny of the various types of ER bodies and their trafficking within a plant cell is shown. The diagram encompasses all of the possible paths and interactions supported by published data. OBs formed by the tER are released into the cytoplasm. The various forms of PB, prolamin storage protein, precursor accumulation vesicles (PAC), and protease precursor vesicles (PPV) are formed by the ER and subsequently are transferred to the vacuole by several different routes including autophagy, binding to the tonoplast, or binding to the tonoplast after addition of Golgi-derived membrane proteins. Except for oil bodies the diverse ER body forms primarily share a similar function to transfer insoluble content of the ER bodies to the vacuole.

aggregates, there is no downstream need for the proteins to self-aggregate in the vacuole matrix. For crystalloids that occupy a substantial portion of the seed PSV matrix, the delivery of already accreted crystalloid protein places concentrated and assembled protein assemblies into the vacuole [46]. The presence of membrane and tonoplast proteins in crystalloids [47] indicates that deposition in the PSV occurs by autophagy.

PAC PBs is another variant that transfers storage proteins directly to the vacuole as large accretions [44,45], perhaps with some contribution from the Golgi [48]. The balance of a soluble, as compared with an accreted, insoluble version of the same protein, presents a number of interesting biological questions. For example, are the sites of synthesis and assembly within the ER network different, depending on the fate of the protein? There are possible test models: soybean mutants and transgenics that lack conglycinin storage proteins accrete a portion of the proglycinin storage proteins into PBs [49,50]. Proglycinin PBs are not present in the wild type, which indicates not only that the lack of conglycinin proteins promotes the formation of stable PBs, but also that these PBs are composed primarily of proglycinin. This indicates that proglycinin is diverted from endomembrane progression to form PBs. Curiously by introgressing another accreted protein, GFP-KDEL in the conglycinin knockdown background impedes the accretion of the proglycinin with the result that it is restored to be transport competent and progresses to the vacuole for processing [51••]. This may suggest that there are protein or chaperone cofactor preferences to accrete one protein in favor of another with the result that accreting one protein, GFP-KDEL, impedes the accretion of another protein, proglycinin [51••]. Another model of soluble and insoluble variants of a protein is zeolin, a fusion protein made of the vicilin, phaseolin, and γ-zein [52]. Zeolin accretes by intramolecular disulfide bonds and forms PBs, while a variant with mutated sites that cannot form intermolecular disulfide bonds is transport competent [53••]. Models such as these will prove useful to understand how otherwise soluble, transport-competent proteins are rendered insoluble and transport incompetent.

A solution for the insoluble?

It seems likely that the paradigm of PB formation by rendering otherwise transport-competent proteins insoluble will prove to be widely distributed in plants. Few of the more than half million plus plant species have been subjected to any cell biological analysis. One hint is the presence of ER bodies in plants that is visualized by expression of green fluorescent protein with a KDEL carboxy-terminal sequence illustrating that there are resident populations of ER bodies in plant cells [54] with unknown functions. This approach could be more widely applied and used to test for the diversity of ER bodies resident in plant cells during growth and development. That chemical stress on the ER will induce the formation of ER bodies is well documented, especially for PBs, and there is now some evidence to indicate that abiotic stress induces ER body formation [42]. Abiotic stress also induces remodeling of the ER, so there is a potential linkage between the effects on ER and ER body formation [42]. With so many types of stress, species variability, nutrient source–sink relationships, and storage compounds, there will probably be many new discoveries about how plants deploy ER bodies and their insoluble contents. Crucial to these discoveries will be an understanding of how plants synthesize and/or convert substances to an insoluble state. By understanding the ontogeny of ER bodies and its relationship to ER function, the biological and physiological processes that induce plants to use ER bodies and the ERVT pathway can be clarified (summary Figure 4).

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This paper shows the movement of proteins within the ER network is much faster that would be expected if solely mediated by diffusion within the lumen. The facilitation of movement has a component that is facilitated by cytoskeletal interactions with the ER. The authors showed that

exit sites from the ER, ER to Golgi in this instance, as discrete sites. The importance of this paper in the context of this review is that it provides parallel experimental data that can be extended to other ER-derived organelles from which can be inferred that ontogeny of other proteins and organelles will also be correlated with the interaction with cytoskeleton.

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This is an important result advancing the correlation of transcripts to specific domains of cytoskeleton and tER to now demonstrating that impeding the association of transcript with the cytoskeleton has the downstream effect of impeding the production of prolamin PBs. The results show that Os-Tudor-SN is a component of the RNA transport particle and is involved in the control of the events leading to prolamin synthesis and subsequent PB formation.

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This paper shows that there are 21 members of the reticulon family grouped into three distinct types. This provides the basis for inferring that different reticulons are expressed to form tER domains with different functions. The results are significant because this raises the possibility that various functions, and especially the export functions, of the ER may be correlated with specific reticulons. With the capacity to do forward and reverse genetics on these 21 members opens up the potential to correlate gene with function of tER domains based on development and physiology.

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This paper is the first demonstration that that mutant or engineered suppression of oleosin alters the size of OBs. Seed OBs are maintained as discrete constant sized organelles through dormancy and desiccation and into rehydration and germination. Oleosins have been assumed to function to maintain the OBs as separate organelles by forming a protein barrier to coalescence. The results of this research show that without an oleosin the OBs merge to form giant OBs.

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This paper also examines the consequences of suppressing oleosin but emphasizes the effects on OB ontogeny and the collateral changes in OB and seed proteome and transcriptome. The oleosin suppressed soybean seeds formed micro OBs that merged to form a hierarchy of OBs contained within an OB/ER complex. The micro OBs appear to be formed from small accretions of TAG forming on the distal tip of the tER. The OBs in oleosin-suppressed seeds accumulates caleosin and in parallel the transcript abundance of caleosin is elevated. The results show that oleosin has a crucial role in forming the initial OB and also indicates that caleosin has a role in OB formation.

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How accreting proteins are manipulated in the ER lumen is an important question and a basis for understanding how PBs are assembled. This paper describes a novel ER membrane protein that has an intralumenal domain that interacts with 22 kDa α-zein. The presence of a protein that has specific interactions with a prolamin is the first evidence and a significant observation that prolamin accretion and PB assembly is at least partly facilitated rather than solely mediated by the hydrophobic interaction, aggregation, and self-assembly. This result may set the stage for further discoveries of ER-localized membrane proteins that mediate the formation of individual members of ER body group of organelles.

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This paper shows that a mutant maize 16 kDa zein with a frame shift in the carboxy-terminal region results informing an aberrant protein that fails to correctly assemble with 22 kDa zein. The result is significant because the mutant protein induces the UPR response with elevated BiP showing that the UPR response does function normally in endosperm recognizing malformed proteins while in parallel not responding to the accretion of prolamins into PBs.

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51. •• Schmidt MA, Herman EM: **Proteome rebalancing in soybean seeds can be exploited to enhance foreign protein accumulation**. *Plant Biotech J* 2008, **6**:832-842.

This paper shows that the capacity to form PBs can be increased by the massive accumulation of a KDEL-tailed GFP model protein where the protein sequestered in the PBs is exchanged for vacuolar storage protein. The results are pertinent to this review by showing the size of the tER generated PB population is plastic and adaptive correlated with the size of the PB-destined protein pool. This paper shows that 25% of the glycinin retained in the ER as proglycinin and forming PBs in a conglycinin knockdown [49] is reduced to 7% proglycinin when GFP-KDEL is introgressed and accumulated to a level of 7–8% of the total protein in PBs. This shows the accretion of one protein, GFP-KDEL, results in transforming much of the transport-incompetent accreted proglycinin to transport-competent glycinin sequestered in the vacuole. The GFP-KDEL therefore impedes accumulation of insoluble proglycinin maintaining its solubility.

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This is a unique paper being the first demonstration that it is the accretion of the protein that leads to the formation of the PB ER bodies. The authors compared the PB forming capacity of a phaseolin/γ-zein fusion protein that aggregates by intermolecular disulfide bond with a variant that is mutated not to form disulfide bonds. The mutant version does not form aggregates and remains transport competent. Although most proteins that accrete to form PBs do not do so by intermolecular disulfide bond formation, the results clearly show that it is making the protein insoluble that is a key step toward producing PBs.

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